4th RMUTIC 2013 organized by Rajamangala University of Technology Phra Nakhon, Bangkok-Thailand

JOURNAL OF APPLIED SCIENCES RESEARCH

JOURNAL home page: http://www.aensiweb.com/jasr.html 2013 Special, 9(12) pages: 6021-6027

Published Online :15 January 2014 **Research Article**

Non-*Saccharomyces* Yeasts as Mutually Dominant Species Involving in Alcohol Fermentation Process

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Received: 12 November 2013; **Revised:** 14 December, 2013; **Accepted:** 20 December 2013.

ABSTRACT

Natural fruit fermentation for alcohol production comprises of various complex microbial activities. Assortments of yeasts and lactic acid bacterial species were sequentially detected during the fermentation process. In the fermentation broth, non-*Saccharomyces* species as well as lactic acid bacteria were firstly carried out for the early stages of the fermentation, although the fermentative *Saccharomyces* yeasts finally became most dominant species. In the study, non-*Saccharomyces* isolates constituted of 50 to 75 % of the total yeast counts were culturable and isolated at the early stages of the alcoholic fermentation commonly. All these non-*Saccharomeces* yeast isolates could affect fast and high alcohol production and outcompete other indigenous yeasts in the fermentation process. By comparison with the nucleotide sequences of the NCBI GenBank Database, these non-*Saccharomyces* isolates, NY3, NY43, and NY55, were genetically identified to be *Kloeckera* sp., *Metschnikowia* sp., and *Hanseniaspora* sp. with their identity of 99-100% homology. In the fermentation test of lychee juice supplemented with glucose, they were all sensitive to high alcohol, surviving not more than 15-18% (vol/ vol) but could survive mutually with *Saccharomyces* spp.

Key words: non-*Saccharomyces* yeast, fermentation, alcohol.

INTRODUCTION

The ethanol production by fermentation has received large importance in the last few years due to its increased demand as fuel and complement to gasoline. It reduces the dependence on oil, the air pollution and climate change caused by emissions of carbon dioxide [4]. Ethanol production usually by fermentation of agro-industrial wastes is very attractive because of abundance and non-competition with foodstuffs [11]. There are dynamics of various indigenous microbe population involved in fermentation. They depend on many factors such as sulphur dioxide, selected yeasts and the interaction with other microorganisms. In addition, mostly it was found that affected microflora are sensitive to climatic conditions (temperature, rainfall, humidity, maritime influences), fruit variety (cultivar, thickness of fruit skin, the composition of the fruit juice), viticultural practices (fertilization, use of fungicides), developmental stage of fruits, and health of fruits (physical damage, insect pests.

Fruit fermentation is not single-species fermentation although the dominance of *S. cerevisiae (*inoculated or indigenous) in the fermentation is desired. Most yeast species were major groups and they were found in different niches associated with fruit growth. The yeasts in alcohol fermentation have been described and arbitrarily divided into two groups, the *Saccharomyces* group and the non-Saccharomyces group. On fruit skins, there is more non-*Saccharomyces* yeasts than *Saccharomyces* group, primary representative, *Saccharomyces cerevisiae*. The non-*Saccharomyces* yeasts typically contain numerous species. They are usually from the cellar surfaces during crushing, especially in the specific environmental conditions that are hostile to them, such as low pH, high sugar (high osmotic pressure), an equimolar mixture of glucose and fructose, presence of SO_2 and a non-optimal growth temperature during cold settling. It was mentioned that more non-Saccharomyces yeast species in the anaerobic environment happened rapidly in spontaneous fermentations [5,3].

The indigenous non-*Saccharomyces* yeasts can adapt to the specific environment and are in an active growth state, giving them a competitive edge. Different factors affect the non-Saccharomyces yeasts are dependent on the characteristics of the individual species. Growth parameters for one

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species will not necessarily be the same for others. Variations can also occur for strains within a species. The majority of non-*Saccharomyces* usually disappear during the early stages of a vigorous fermentation due to their slow growth and inhibition by the combined effects of SO_2, low pH and high ethanol content [3]. It has been documented that the species that are survive and present until the end of fermentation may also have a higher tolerance to ethanol. For example, *C. stellata* can tolerate up to 12% ethanol [3]. The role of these yeasts in fruit fermentation, although carrying a higher risk of spoilage, are generally regarded as having improved complexity, mouth-feel (texture) and integration of flavors [15]. Different non-*Saccharomyces* yeasts produce different levels of higher alcohols (n-propanol, isobutanol, isoamyl alcohol, active amyl alcohol) [10]. Spontaneous fermentations (comprising mixed cultures of non-*Saccharomyces* and *Saccharomyces* yeasts) produce superior wines compared with pure culture fermentations. However, growth of non-*Saccharomyces* yeasts can be limited by the *S. cerevisiae* starter culture. High concentrations of S. cerevisiae appear to inhibit some non-*Saccharomyces* yeasts by means of a cell-cell mediated mechanism [13].

Therefore from overall, we were interested in understanding the characteristics of these mutual dominant non-*Saccharomyces* yeasts species in early alcohol fermentation process. The possibly culturable isolates compared with *Saccharomyces* yeasts in alcohol fermentation and their advantageous utilization to increase the fermentation quality would be observed.

Methodology:

Microorganisms:

All strains of isolated non-*Saccharomyces* yeasts (culturable strains of NY3, NY43, and NY55) and S. cerevisiae (EC1118) were pre-cultured under aerobic condition at 30^{o}C for 24 h and shaking at 160 rpm in 3ml of YPD medium containing 10 gL^{-1} yeast extract, 20 gL^{-1} peptone, and 20 gL^{-1} glucose. Cell were later diluted to OD_{600} equal to 0.4 in YPGS medium and used as seed cultures in the fermentation broth of small scale fermentation.

Small-scale alcohol production:

In the fermentation test for the activity of non-*Saccharomyces* yeast, the NY yeast cultures (propagated in YPD broth) were inoculated individually at 1 x 10^6 cells mL^{-1} (0.4 OD_{600}) in a batch scale of 5 L. Other alcohol production treatments were according to the standard procedures for small-scale alcohol production [7]. During fermentation, 20 mL aliquots were regularly removed for reducing sugar analyses as well as other parameters such as pH and cell numbers of microbes were measured.

Conventional alcohol fermentation process:

Spontaneous fermentation was performed by conventional fermentation [8]. It included mechanical digestion by crushing and squeezing for lycheeee juice. The lychee juice was thus filtrated before reducing sugar adjustment and further incubated at 20^{o}C under anaerobic condition for small-scale production of alcohol in aliquots of 18 L. Non-*Saccharomyces* yeasts in combination with lactic acid bacteria and *S, cerevisiae* were investigated. During the fermentation period, total reducing sugar, pH and microbes were recorded. The acidity representing as pH and ethanol concentration together with the cell numbers of non-*Saccharomyces* yeasts were estimated every 2 days. The cell concentration was measured at OD_{600}, using a spectrophotometer (BioSpec-1600, Shimadzu, Kyoto, Japan) and alcohol determination by using an alcohol densitometer (Alcomate AL-2, Riken Keiki, Kyoto, Japan). All estimations were performed in triplicate, and the average values were applied for the study.

Growth of cell culture and DNA extraction:

The isolated cells were subjected for DNA extraction. They were grown for approximately 24 h at 25°C in 50 ml of YM broth (3 g yeast extract, 3 g malt extract, 5 g peptone, and 10 g glucose per liter of distilled water) on a rotary shaker at 200 rpm and harvested by centrifugation. The obtained cells were washed once with distilled water, resuspended in 2 ml of distilled water and aliquoted to two 1.5- ml microcentrifuge tubes. After centrifugation, excess water was decanted from the microcentrifuge tubes, and the packed cells were freeze-dried for 1–2 days and stored at–20°C until used. DNA isolation for polymerase chain reaction (PCR) was performed using the CTAB (hexadecyltrimethyl-ammonium bromide) procedure that was described in detail by Raeder and Broda,1985.

PCR amplification and species identification:

The oligonucleotide primers for symmetrical amplification of gene sequences and sequencing of the genes compared are ITS1–5.8S–ITS2 *rDNA* given in the following sections. Primers for symmetrical amplifications were various combinations of the following: ITS-5 ′(5′-GGAAGTAAAAGTCGTAACAAGG) (F), ITS-4 (5′-TCCTCCGCTTATTGATATGC) (R). Temperatures for PCR were 52°C annealing and 72°C extension or 39°C annealing and 60°C extension. Sequencing primers were ITS2 ′(5′-GCTGCGTTCTTCATCGATGC) (R), and ITS-3 (5′-

GCATCGATGAAGAACGCAGC) (F). The symmetrical amplifications were performed for 36 PCR cycles with denaturation at 94°C for 1 min, annealing at 52°C, and extension at 72°C for 2 min, with the final extension for 10 min [9]. Both strands of the DNAs compared were sequenced with the ABI TaqDyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) using either ABI 377 (gel) or ABI 3100 (capillary) automated DNA sequencers, following the manufacturer's instructions. The unambiguously were aligned nucleotide sequences by comparing with the nucleotide sequences GenBank database with the homology of 99-100%. They were all classified for their accession numbers as AJ512455, FJ873430, and AY796025 that were classified to be *Kloeckera* sp., *Metschnikowia* sp., and *Hanseniaspora* sp.

Results:

Non-Saccharomyces yeast populations involving in alcohol fermentation:

There were differences in microbe populations between two tests, one composed of lactic acid bacteria, non-*saccharomyces* spp., and *Saccharomyces* sp. in spontaneous, natural, fermentation test and the other only inoculated with *Saccharomyces cerevisiae* at the early phase. In 24 days, both tests showed the cell growth following the common patterns of lag, log, and stationary phases but no decline phase was determined (data not shown). Microscopic observation, showed that natural alcoholic fermentation of lychee juice created an environmental niche of various natural lactic acid bacteria, non-*S. cerevisiae* yeasts and *Saccharomyces* yeasts. These various main fermentable strains involved by the sequential development of various species including bacteria, fungi, and yeasts, example shown in figure 1. By microscopic observation, the high cell numbers of yeasts (1-15X10^{12} cells ml^{-1}) and bacteria (5 X 10^{11}-25 X 10^{12} cells ml^{-1}) were presented all over in the broth. More bacterial and non-*Saccharomyces* yeast cells were found in the log phase that were accounted for the early phase, whereas more *S. cerevisiae* cells were later detected in the late phase (data not shown). These microbial communities produced high yeild alcohol at 18-20% (vol/vol) in the fermentation of lychee juice. In the study of fermentation for a month, similar increase patterns of the alcohol production to the growth curves but oppositely to the percentages of reducing sugar utilization that were decreased (figure 2). They gave consistently high ethanol content increase to 18-20% (vol/ vol) whereas of only *Saccharomyces* yeasts showed the alcohol content gradually increase to 15-18% (vol/vol). More bacterial and non-*Saccharomyces* yeast cells were found in the log phase that were accounted for the early phase, whereas more *S. cerevisiae* cells were later detected in the late phase (data not shown). The species of non-*Saccharomyces* were identified for the species by comparison their nucleotide sequences of ITS1-5.8S-ITS2 genes with those in the GenBank database. Their homology of their molecular analysed ITS genes at 99%, 99.99% and 100% were *Kloeckera* sp., *Metschnikowia* sp., and *Hanseniaspora*, respectively.

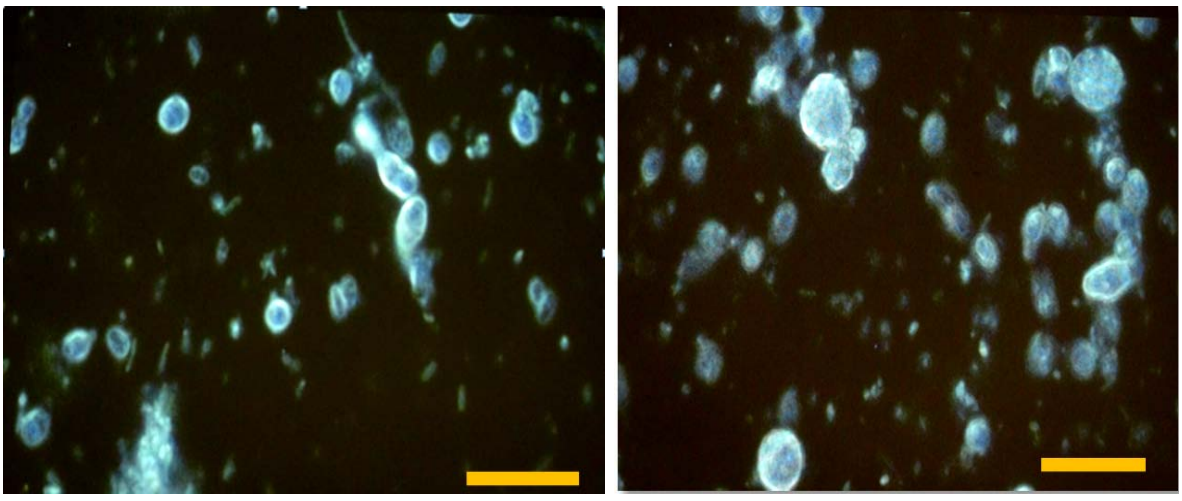

Fig. 1: Two examples of natural indigenious non-*Saccharomyces* and bacteria during the initial fermentation stage observed by microscopy. Many bacteria and Yeasts species were sequentially developed at the early fermentation period. The bar shown is 20 μm.

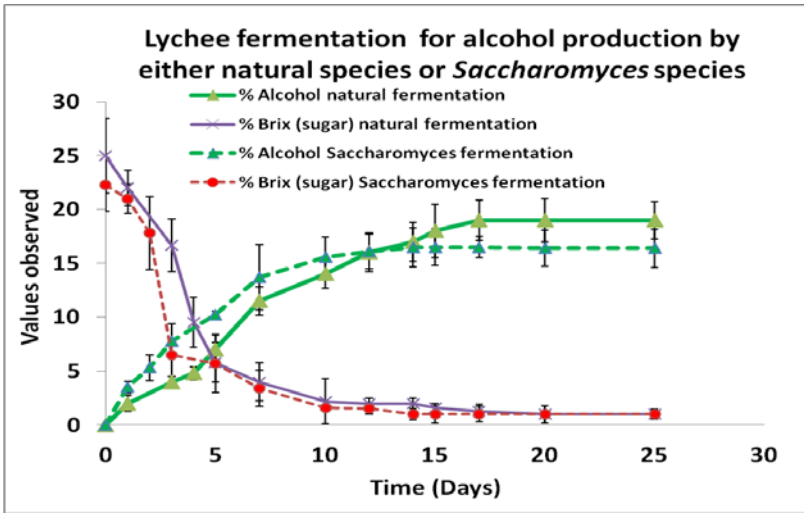

Fig. 2: Comparison of lychee fermentative conditions produced by either natural, indigenous microbial communities (composed of lactic acid bacteria, non-*saccharomyces* spp. and *Saccharomyces* sp.) or *Saccharomyces* (EC1118), fermentation. The fermented cultures were incubated for 24 days and all conditions including pH, % reducing sugar and total sugar consumption were regularly determined.

These dominated non-*Saccharomyces* yeasts isolates were obtained from the lychee fermentation at the early stage by selective culturing. Three isolates (NY3, NY43, and NY55) from total 36 isolates of non-*Saccharomyces* yeasts were obtained by plating and culturing in YEPD media at the early stage of the alcoholic fermentation. NY3, NY43, and NY55 were later identified to be species *Kloeckera* sp., *Hanseniaspora* sp., and *Metschnikowia* sp. By microscopy, these isolates were pure, non-motile and had big sizes of 5-15 um. NY43 cells were bigger than NY3 but both of them were round whereas NY55 cells were oval. The microscopic observation was shown in figure 3.

The pure culture of either NY3 isolates or *Saccharomyces* cerevisiae were inoculated in the fermenation of lychee juice supplemented with glucose at 25% Brixs. Both of them could produce high contents of alcohol saturated at 15-18% (vol/ vol). In 25 days, although better alcohol production was observed from the *Saccharomyces* yeast sample, both samples displayed very similar sugar utilization patterns that were fast dropped at day 5. It was possible that NY3 used nutrients for maintaining their cell growh. Certain NY3 cells were earlydropped during high alcohol concentration (10-14% (vol/ vol) due to the alcohol toxicity, however, after 12-15 days, the cells showed better saturated alcohol production to 18% (vol/ vol). The results were shown in figure 4. Moreover, in further observation, saturated alcohol content from NY3 was reached at 15-18 % (v/v) in which it was lower than that from *Saccharomyces* yeasts. These NY3 cells started to die after two week fermentation since undetectable cells was seen under microscope (data not shown).

Discussions:

The importance of non-Saccharomyces yeasts in the early stage of alcohol fermentation:

In the early lychee fermentation process having the environment of high sugar contents (20-25% Brixs), all microbes had adapted and mutiplied. Their activities involved in converting all compounds to sugar moieties as well as further producing alcohol. Non-*Saccharomyces* yeasts (NY) as epiphytic microflora were essentially responsible for the early stages of alcohol fermentation. At this stage, these non-*Saccharomyces* species firstly dominated bacteria, possibly due to their habitating on surface of lychee. However, *S. cerevisiae* were high resistance to alcohol but also stronger in competing for the nutrients than non-*Saccharomyces* yeasts since after the fermentative process was approximately 4-5 days, non-*Saccharomyces* yeasts started to decay rapidly as ethanol concentration increased, and were replaced by indigenous *S. cerevisiae* strains (data not shown). They were sensitive to the present of high alcohol concentration. Their high activity could be prolong in the present alcohol not above 10-12% (vol/ vol). In addition, the fermentation period should not be longer than fifteen days and then the *S. cerevisiae* could be inoculated to maintain prolong high alcohol production. Non-*Saccharomyces* yeasts would begin fermentation before the populations of*Saccharomyces* yeast have established a sufficient cell density to overwhelm the non-*Saccharomyces* yeast. They could be able to ferment wine until the alcohol content reaches 10-15%, where their sensitivity to ethanol prevents further metabolism and causes a die off of all these yeast species.

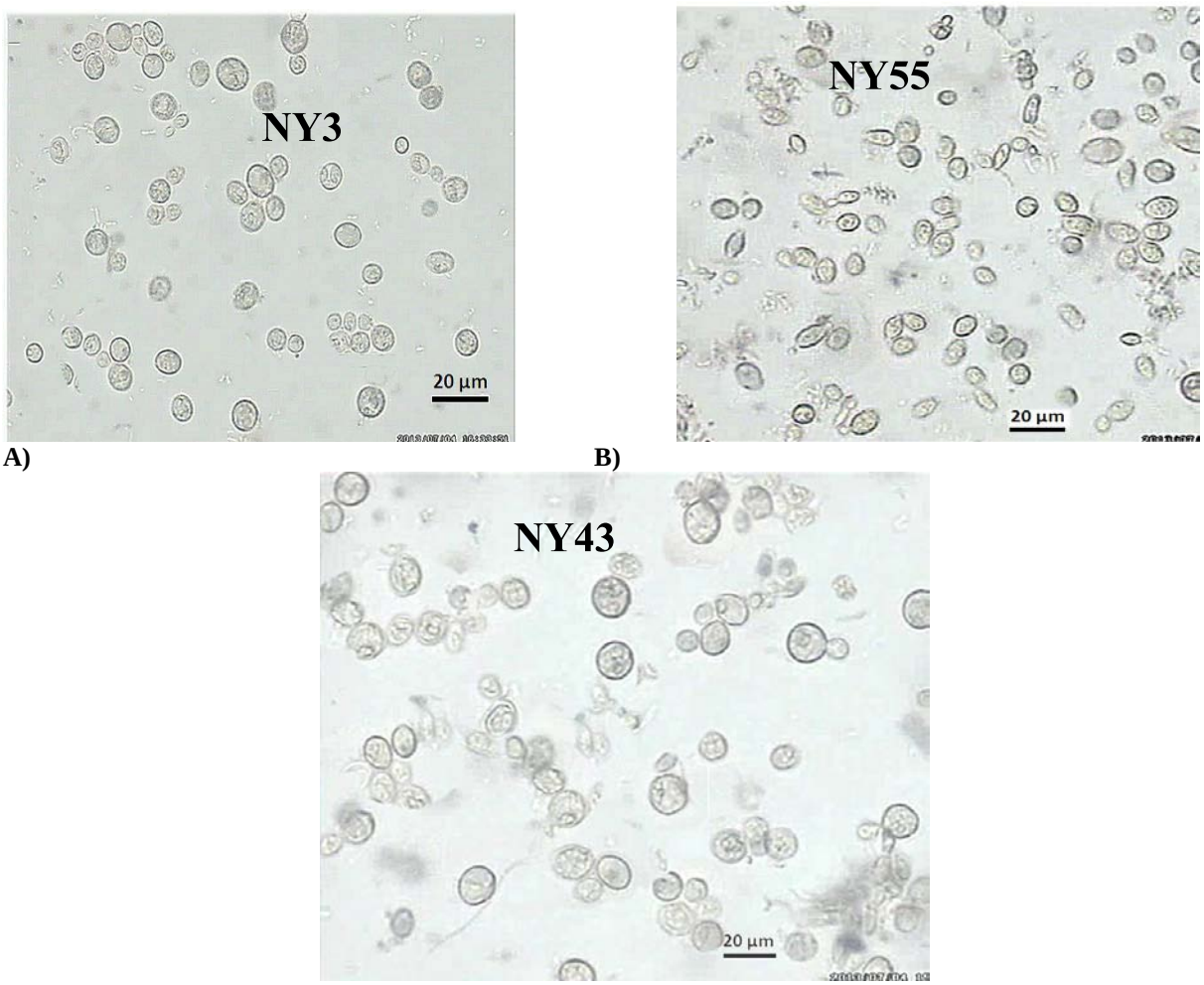

Fig. 3: The microscopic observation of non-*Saccharomyces* yeasts (NY) isolated from the lychee fermentation at the early stage. According to Genebank database of ITS1-5.8S-ITS2 nucleotide sequences, three non-*Saccharomyces* isolates, NY3 (A), NY43 (B), and NY55 (C) were identified to be *Kloeckera* sp., *Hanseniaspora* sp., and *Metschnikowia* sp.

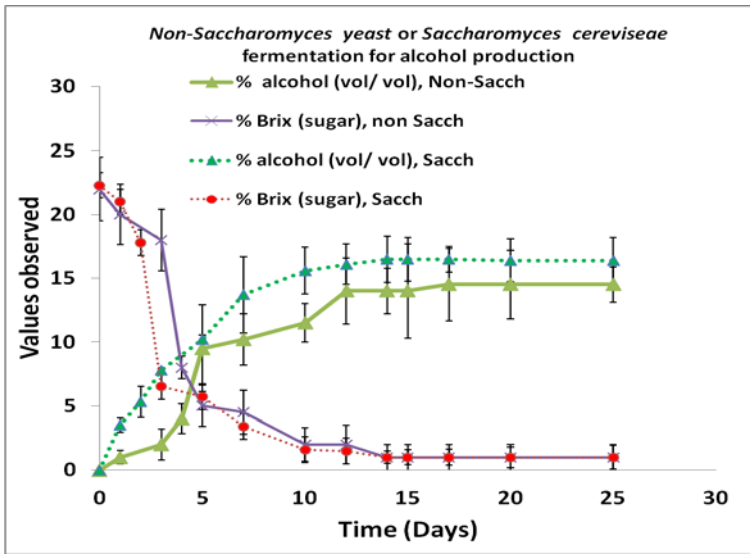

Fig. 4: Inoculated pure culture of either non-*Saccharomyces* isolate, NY3 or Saccharomyces yeast (EC1118), (1 X 10^{10} cells ml^{-1}), in lychee juice fermentation. The alcohol concentration, total acidity, and cell concentrations were measured regularly for 3 weeks. Alcohol production was rapidly increased in correlation with more sugar utilization whereas the cell growth was increased to 1 X 10^{14} cells.

Non-Saccharomyces yeasts were weaker than Saccharomyces because they began fermentations faster to help to prevent other unfavorable organisms from becoming established prior to the end of the lag period of *Saccharomyces*. Saturated alcohol content thus reached only at very low concentration (15-18 % (v/v) by NY3 activity) and evenmore, after two month fermentaion, these yeasts were very low, undetectable under microscope. *S.cerevisiae* could better stand in the fermentative broth for more than 5 moths (data not shown) but in the present of non-Saccharomyces yeasts, the high alcohol yield at shorter time could be done. It has been reported by non-*Saccharomyces* yeasts tend to be viewed as favorable to fermentations to allow for added flavor to the wine [15]. Therefore, it will be better to apply the combination of non-Saccharomyces with common *S. cervesiae*, especially as sequential inoculating cultures in the fruit juice fermentation.

In the fermentation broth of lychee juice, both yeasts demonstrated similar patterns of alcohol production and sugar utilization. It was possible that these non-*Saccharomyces* yeast isolates (NY) could alone produce alcohol but it would be better to be adding them first and followed by *Saccharomyces* yeast. These results were different from the work by Jolly et al. 2003. The report demonstrated that two different inoculation protocols of co-inoculation and sequential inoculation with non-*Saccharomyces* yeasts (*C. stellata*) and *S. cerevisiae* showed total differences in their growth patterns in fermentation process [7,8]. In the sequential inoculation, it was found that the early death of some non-*Saccharomyces* yeasts during fermentation could also be a source of specific nutrients for *S. cerevisiae*, enabling it to ferment optimally. There are evident that their nutrients include cellular constituents such as cell wall polysaccharides [5].

Isolated non-Saccharomyces and its characteristics:

Non-*Saccharomyces* such as NY3 isolate or *Kloeckera*, had their fermentative capacity and high resistance to high ethanol concentration without combination with a natural *S. cerevisiae*. They could utilize sugar as well as resist high acidity but they were significantly less efficient and weaker than *S. cerevisiae* in converting sugar into ethanol. As recorded by Jolly et al [8], the combination of non-*Saccharomyces* yests and *S. cerevisiae* communities could decrease fermentation period and faster sugar utilization were detected. They affected to alcohol quality in that they increased the total acidity, the faster expected values of pH and alcohol production at shorter time. These native residing non-*Saccharomyces* yeasts have influenced the spontaneous fermentation capacity in that they can survive long and function in concert with commercial *Saccharomyces* species in production of ethanol. Non-*Saccharomyces* yeasts could not able to certainly sustain their presence a short time whereas there were increasing ethanol concentrations produced by *Saccharomyces* species. The increasing concentration of ethanol and other fermentation products possibly caused the non-*Saccharomyces* died [15]. Therefore, from the study, NY isolates should be further benificially applicable in combination with S. cerevisiae in high alcohol production with good aroma and quality.

Conclusions:

The development of the different yeast strains affecting the alcohol production could be optimized by monitoring isolated indigenous microbiota. They can be a selective good choice for further purposes of fermentation starters. As evident from the study, the communities of indigenous non-*Saccharomyce* yeasts with their contributions have clearly influenced in overall fermentation periods. Non-*Saccharomyces* species play a major role in the initial phase of fermentation, while the commercial *S. cerevisiae* yeast was desired for completion of fermentation and production of a specific alcohol style. Although these isolated strains are dominant species in the microbial population and this study was only semiquantitative approach in determining the characteristics of a small percentage of all non-Saccharomyce yeast populations, it is possible that many unculturable species of non-*Saccharomyces* as well as *Saccharomyces* spp. can possibly take charge in the fermentation. Moreover, in natural, the successive changes of yeast population compositions occurred were mainly connected with the increase of ethanol concentration and decrease of nutrient components. The phenomena of microbial assortments in the fermentative environment shall be on a regular basis exploited to explain for several natural occurrences such as acidity, total sugar consumption, and ethanol yields, during the fermentation process.

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16. Acknowledgements: The authors wish to thank the Rajamangala University of Technology Phra Nakhon (RMUTP) for kindly support in this work.